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RESEARCH ARTICLE

ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties

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Herpes simplex virus type-1 (HSV1) in which the neurovirulence factor ICP34.5 is inactivated has been shown to direct tumour-specific cell lysis in several tumour models. Such viruses have also been shown to be safe in Phase I clinical trials by intra-tumoral injection in glioma and melanoma patients.[1–3] Previous work has used serially passaged laboratory isolates of HSV1 which we hypothesized may be attenuated in their lytic capability in human tumour cells as compared to more recent clinical isolates. To produce ICP34.5 deleted HSV with enhanced oncolytic potential, we tested two clinical isolates. Both showed improved cell killing in all human tumour cell lines tested compared to a laboratory strain (strain 17+). ICP34.5 was then deleted from one of the clinical isolate strains (strain JS1). Enhanced tumour cell killing with ICP34.5 deleted HSV has also been reported by the deletion of ICP47 by the up-regulation of US11 which occurs following this mutation.[4,5] Thus to further improve oncolytic properties, ICP47 was removed from JS1/ICP34.5–. As ICP47 also functions to block antigen processing in HSV infected cells, this mutation was also anticipated to improve the immune stimulating properties of the virus. Finally, to provide viruses with maximum oncolytic and immune stimulating properties, the gene for human or mouse GM-CSF was inserted into the JS1/34.5–/47– vector backbone. GM-CSF is a potent immune stimulator promoting the differentiation of progenitor cells into dendritic cells and has shown promise in clinical trials when delivered by a number of means. Combination of GM-CSF with oncolytic therapy may be particularly effective as the necrotic cell death accompanying virus replication should serve to effectively release tumour antigens to then induce a GM-CSF-enhanced immune response. This would, in effect, provide an in situ, patient-specific, anti-tumour vaccine. The viruses constructed were tested in vitro in human tumour cell lines and in vivo in mice demonstrating significant anti-tumour effects. These were greatly improved compared to viruses not containing each of the modifications described. In vivo, both injected and non-injected tumours showed significant shrinkage or clearance and mice were protected against re-challenge with tumour cells. The data presented indicate that JS1/ICP34.5–/ICP47–/GM-CSF acts as a powerful oncolytic agent which may be appropriate for the treatment of a number of solid tumour types in man.
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Introduction

Oncolytic viruses selectively replicate in, and kill, tumour cells and as such have shown considerable promise for the treatment of cancer (reviewed by Hawkins et al[6] and Ring[7]). Oncolytic virus therapy has a number of potential advantages over other gene-based approaches to cancer treatment in that virus replication will not only directly kill tumour cells, but will also disseminate the therapeutic agent further through the tumour tissue than non-replicating therapies. Thus, if the oncolytic viruses were also to carry a therapeutic gene, the efficiency of gene delivery to the tumour should be enhanced as compared to gene vectors which do not include a replicative component.

To date, although gene therapy for cancer has often given promising results in animal models, the results of clinical trials have generally been disappointing. This is probably due to the fact that gene delivery is only accomplished in a small portion of the tumour tissue and accompanying problems with the large vector doses then required to give significant gene transfer. Non-replicating vectors based on adenoviruses and retroviruses have been tested in the clinic for direct intra-tumoral delivery of potentially therapeutic genes such as p53 (reviewed by Fujiwara et al[8]), various pro-drug activators such as thymidine kinase and nitroreductase (reviewed by Xu and McLeod[9]), or immune stimulatory molecules such as IL2 (eg references 10,11) or various interferons (reviewed by Ferrantini and Belardelli [12]). Significant efficacy has not, however, so far been demonstrated. Intra-tumoral administration of naked DNA or DNA in liposome formulations to deliver similar genes has also been attempted, but without clinical success, probably due to the very low gene transfer efficiency achieved (eg see reference 13).

Taken together, these results indicate that if gene therapy for cancer via direct intra-tumoral injection is to be clinically effective, greater gene transfer efficiencies are required. Such an improvement may be provided through the use of replicative vectors.

Various oncolytic viruses have been developed based on viruses including adenoviruses, herpesviruses (HSV), reoviruses, retroviruses, and more recently VSV and measles virus (oncolytic virus therapy reviewed by Hawkins et al[6,7]). These generally exploit the fact that for a virus to replicate in a target cell, cellular antiviral response pathways must usually be disabled. Viruses such as herpesviruses and adenoviruses encode genes to block the antiviral response and allow replication, whereas other viruses such as reoviruses and VSV cannot generally replicate in human cells (although they can infect), as they do not encode anti-viral response-blocking genes. As tumour cells often have a disabled anti-viral response pathway, viruses such as reoviruses and VSV can replicate in tumour cells but not normal cells where replication is blocked. Likewise, the removal of anti-viral response avoidance genes from viruses such as adenoviruses and herpesviruses provides the property of tumour-selective replication as replication is then blocked in normal cells (see below). Alternatively, tumour-selective replicative viruses have been developed through the use of tumour-specific promoters to drive the expression of an essential virus gene only in the tumour. Clinical testing using this approach includes the use of adenoviruses, where the E1A region of the virus is driven by a prostate-specific promoter.[14]

Clinical testing with viruses in which pathways blocking the anti-viral response have been disabled has used adenoviruses in which the E1B region has been deleted (ONYX-O15)[15] and herpesviruses in which the ICP34.5 gene (the so-called 'neurovirulence factor') has been removed (1716 and G207 [G207 is also deleted for the ICP6 gene encoding ribonucleotide reductase necessary for growth in neurons but dispensable in other cells][16,17]). The adenovirus E1B region is required for virus replication in cells with a functional p53 pathway, but is dispensable in most tumour cells where the p53 or related pathways are disrupted (eg see reference 18). The HSV ICP34.5 gene counteracts the interferon-induced PKR-mediated block to virus replication,[19,20] which is usually disabled in tumour cells. ONYX-O15 has been tested in a variety of clinical situations with maximal success following repeated administration at high dose in head and neck cancer patients [21–23] or intra-hepatic artery infusion into liver cancer patients,[24] both combined with chemotherapy. 1716 and G207 have both been tested in glioma patients with some evidence of a slowing of the progress of the disease (1716).[1,2] 1716 has also been tested at very low dose in melanoma patients.[3]

The work reported in this paper aimed to build on previous work on oncolytic HSV, which has shown clinical promise, but would likely benefit from enhanced anti-tumour potency. Specifically, a recombinant virus was constructed which gave enhanced oncolysis through the use of a more potent clinical isolate of HSV, rather than the laboratory strains previously used, and incorporation of a mutation increasing the expression of the HSV US11 gene. Increased expression of US11 has

previously been shown to enhance replication of HSV ICP34.5 mutants in tumours.[25–27] Additionally, the gene for GM-CSF was inserted to maximize the immune response generated following the release of tumour antigens by virus replication. This immune response was further enhanced as the method of increasing US11 expression also deletes the ICP47 gene which usually blocks antigen presentation in HSV infected cells.[28] Such an approach aims, therefore, to provide an in situ, tumour-specific vaccine, the efficacy of which is enhanced by accompanying virus replication. This paper describes the development and pre-clinical testing of this virus which has received approval to begin clinical trials in the UK during 2002.

Results

Virus construction and characterization

HSV1 strain 17+ has been described previously. ICP34.5 was deleted from strain 17+ together with the insertion of a GFP marker gene as described in Materials and Methods. HSV1 strains BL1 and JS1 were isolated from cold sores from reactivating individuals and were first confirmed as HSV1 using an HSV1-specific ELISA (not shown). Small stocks of these viruses were then produced and used for the experiments shown in Figure 2a. Viral DNA was prepared from strain JS1, and the ICP34.5 and ICP47 genes were deleted. Human or mouse versions of GM-CSF were then inserted. Strain 17+-derived plasmids were used for this manipulation as strain 17+ provides the only currently available fully sequenced strain of HSV1. However, as described below, the sequence of strain JS1 currently available is very similar to that of strain 17+, as would be expected for any strain of HSV1. Following construction of the set of viruses shown in Figure 1a, Southern blots were performed to confirm the structure of the viruses, and restriction profiling was performed to show the general similarity of the JS1 sequence to a reference strain of HSV1 (strain 17+) and lack of similarity to a reference strain of HSV2 (strain HG52; Figure 1b). The stability of the final virus (JS1/34.5–/47–/hGM-CSF) with serial passage over time was also confirmed (not shown). GM-CSF expression was demonstrated by ELISA in BHK cells and in tumour extracts (see below). Since the thymidine kinase gene has been left intact in the viruses constructed, sensitivity to acyclovir was confirmed in vitro using strain 17+ as a control where essentially identical results were obtained (not shown). Sequencing of the regions altered in JS1/34.5–/47–/hGM-CSF was also performed, resulting in approximately 10 Kb of sequence and confirming the alterations made. Sequencing of the entire JS1 genome is underway. This has so far demonstrated that the JS1 sequence is similar to strain 17+ (see Table 1). The complete DNA sequence of HSV1 strain JS1 will be presented elsewhere.

Use of ICP34.5. deleted clinical isolates of HSV improves tumour cell killing

Previous versions of HSV which selectively replicate in tumours have been based on serially passaged strains of HSV1. Such viruses based on HSV1 strain 17+ or strain F deleted for ICP34.5 (virus strain 1716) or ICP34.5 and
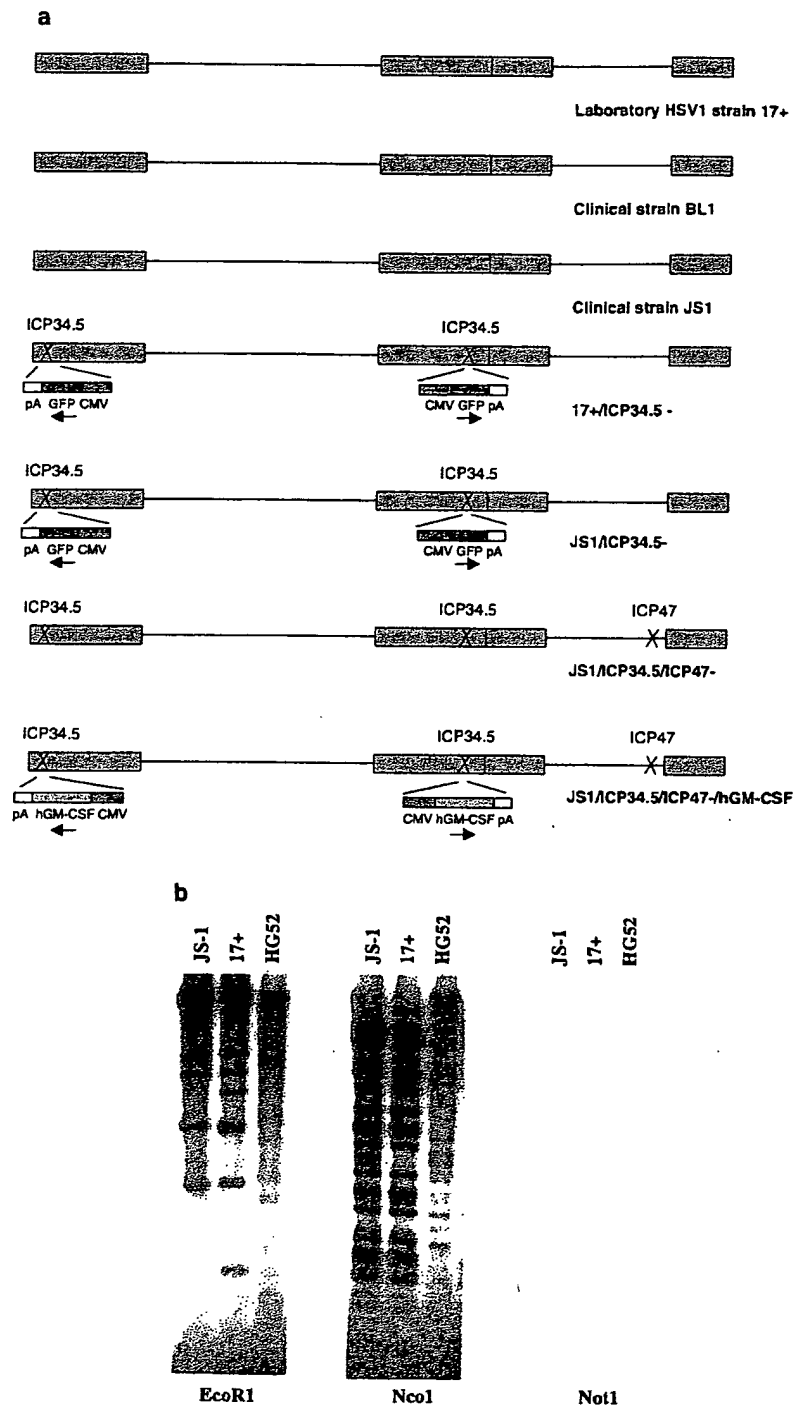
a

b

Figure 1 Viruses used, and comparison of JS1 to HSV1 strain 17+ and HSV2 strain HG52. (a) Schematic representation of the viruses used in the study; (b) restriction profiles of JS1, HSV1 strain 17+ and HSV2 strain following digestion of genomic DNA isolated from each virus with the restriction enzymes indicated (see Methods).

ICP6 (virus strain G207), respectively, have been tested in early stage clinical trials in glioma or melanoma (see Introduction).

We hypothesized that the use of strains of HSV, which have been serially passaged in culture in non-human cells for many years, was likely to have resulted in attenuation such that the full oncolytic potential of the viruses to (i) infect and replicate in human cells and (ii) spread in solid tissue such as a tumour would have been reduced.

Table 1 Summary of sequence comparisons between HSV1 strains 17+ and portions of HSV1 strain JS1 so far sequenced

Region sequenced	Fragment size (bp)	Strain 17+nucleotide numbers	Sequence differences outside flanking region used for gene deletion	Sequence differences within flanking region used for gene deletion
ICP34.5 Fragment1	2022	122858–124863	2 single amino acid changes in ICP0 2 non-coding base changes	2 non-coding base changes 1 silent codon change 15 bp insert in non-coding region
ICP34.5 Fragment 2	3039	125723–127789		10 non-coding base changes
ICP47 Fragment	5229	142003–147733	2 amino acid changes in US8 gene; 9 silent codon changes in US8; 1 amino acid change in US8a gene; 3 silent codon changes in US8a gene; 2 amino acid changes in US9 gene 2 silent codon changes in US9 gene; 7 non-coding base changes; 2 amino acid changes in RS1 gene 1 silent codon change in RS1 gene	21 non-coding base changes; 3 deletions of repeat units; 2 insertions in repeat units

To test this hypothesis, two clinical strains of HSV1, strains BL1 and JS1, were isolated from cold sores from otherwise healthy volunteers (see above) and tested for tumour cell killing in vitro as compared to a standard laboratory strain of HSV1, strain 17+. 17+ is the most virulent of the strains of HSV on which oncolytic versions of the virus have previously

been based,[29,30]. and thus strain 17+ was chosen as an appropriate control against which to compare. This showed (Figure 2a) that in all the tumour cell lines tested, both BL1 and JS1 gave superior tumour cell killing as compared to strain 17+. Here tumour cells were killed more quickly as well as with a lower dose of virus than when using HSV1 strain 17+. It can
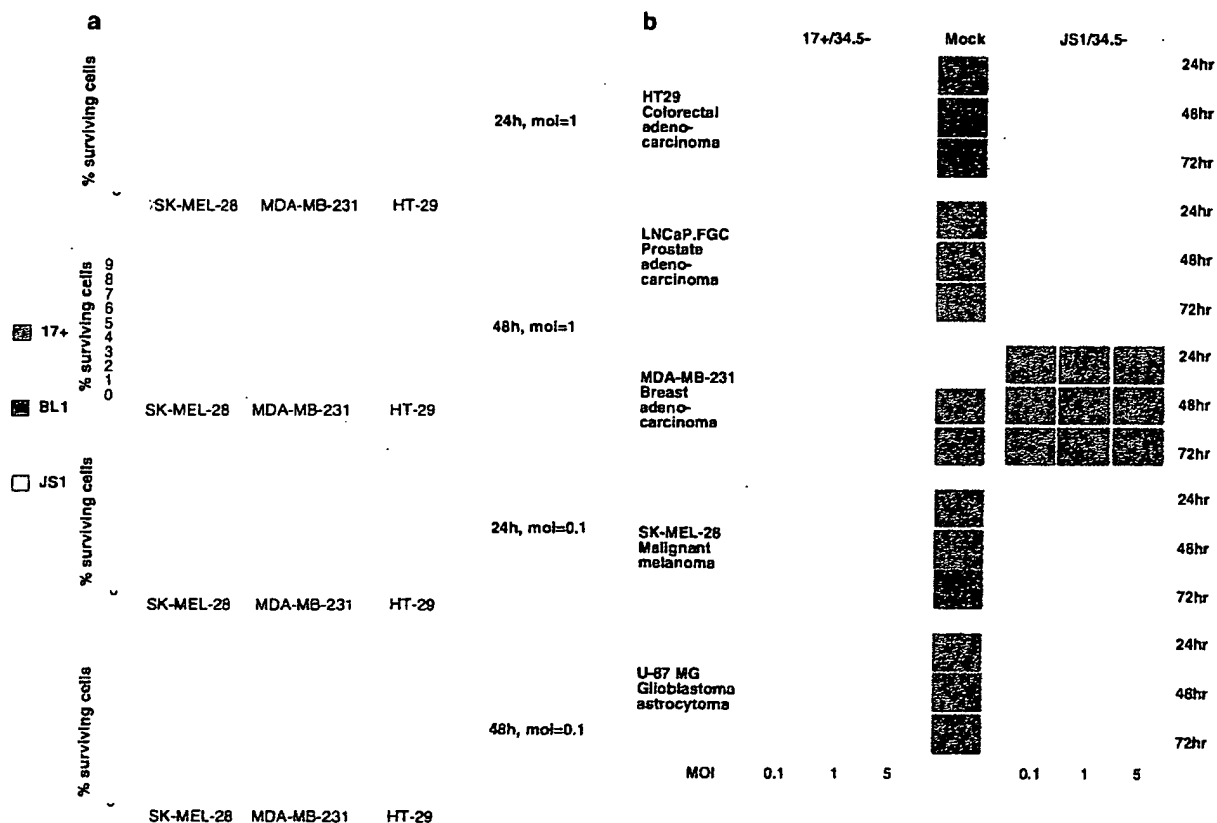

Figure 2 Use of clinical isolates of HSV improves tumour cell killing in vitro. (a) Virus strain BL1, JS1 and 17+ were used to infect the indicated tumour cell lines at the indicated MOIs and cell survival assessed over time by trypan blue exclusion assay; (b) JS1/ICP34.5– and 17+/ICP34.5– were used to infect the indicated tumour cells at the indicated MOIs and at the times indicated. Monolayers were then stained with crystal violet and results photographed.

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be seen from Figure 2a that strain JS1 gives generally superior tumour cell killing to strain BL1. Thus, different clinical isolates of HSV1 behave differently with regard to tumour cell killing, and as JS1 was found to be superior, strain JS1 was chosen for further development.

To generate a version of JS1 with the property of tumour selective replication, the gene encoding ICP34.5. was deleted from strain JS1, and also from strain 17+ as a control (see above). These viruses were then again compared for tumour cell lysis in vitro (Figure 2b). This showed that in all cases tested JS1/ICP34.5− had superior tumour cell killing properties as compared to 17+/ICP34.5−. From this we concluded that the use of an oncolytic version of HSV based on a recent clinical isolate, rather than the serially passaged laboratory strains used previously, is likely to improve the tumour cell killing properties of the virus when used for tumour treatment in vivo.

JS1/34.5− has growth properties typical of an HSV ICP34.5 deleted virus

To confirm that deletion of ICP34.5 from JS1 provided a phenotype typical for an HSV1 ICP34.5 deletion mutant, JS1/ICP34.5− and 17+/ICP34.5− were tested for growth on cells which are known to support the growth of wild-type HSV, but which do not support the growth of HSV ICP34.5 mutants (3T6 cells) [31] as compared to wild type strains JS1 and 17+ (Figure 3). This demonstrated that neither JS1/ICP34.5− nor 17+/ICP34.5− grew on these cells, whereas abundant growth occurred with wild-type strains JS1 and 17+. To further confirm that deletion of ICP34.5 from JS1 provided attenuation typical for an ICP34.5 deleted strain of HSV1, Balb/c mice were inoculated in the flank (s.c.) with multiple high doses of the virus (every other day three times with $1 \times 10^9 \times$ pfu of JS1/34.5−) with no ill effects. Additionally, 20 mice were inoculated by the intra-cerebral route (i.c.) with up to 1×10^5 pfu/ml. This was generally well tolerated. However, two animals did show signs of sickness, similar to previous work with ICP34.5 deleted HSV1 strain 17+ inoculated i.c. which concluded that this resulted from an inflammatory effect.[32] The i.c. LD50 of wild-type HSV1 strain 17+ is <10 pfu.[16]

Increased expression of US11 improves tumour destruction in vivo

It has previously been reported that mutations which alter the regulation of the HSV US11 gene such that it is expressed as an immediate early (IE) rather than a late

(L) gene improve the tumour cell killing properties of the virus without affecting the reduction of pathogenicity in vivo provided by the ICP34.5. mutation.[5,33] US11 blocks PKR phosphorylation and expression as an IE gene allows this to occur before PKR phosphorylation would otherwise have been induced by virus infection.[34,35]

To further improve the tumour cell killing properties of JS1/ICP34.5− the IE gene ICP47 gene was deleted such that the US11 coding sequence was placed under the control of the promoter which usually regulates the expression of ICP47. US11 would then be expected to be regulated as an IE rather than an L gene. ICP47 usually blocks antigen presentation in HSV infected cells,[28] so this mutation was also anticipated to improve the immune stimulating properties of the virus (see below).

To test whether altered regulation of US11 affected the tumour killing properties of JS1/ICP34.5−, the additionally modified virus (JS1/ICP34.5−/ICP47−) was tested in a number of mouse xenograft models in vivo in comparison to JS1/ICP34.5−. Here tumours were generated in the flanks of nude mice, and either injected three times with vehicle, with JS1/34.5−, or with JS1/34.5−/47−, and effects observed. As the experiments were performed with human cells in immuno-compromised (nude) mice, any differences observed were anticipated to relate to the alteration in US11 regulation rather than immune effects associated with the lack of expression of ICP47. This was also anticipated to be the case as ICP47 does not function in mice.[36]

Figure 4 shows the effects of the mutation in three tumour models. In each case it can be seen that the anti-tumour effects are enhanced and more prolonged with JS1/ICP34.5−/ICP47− as compared to JS1/ICP34.5−.

Removal of ICP47 improves antigen presentation

ICP47 blocks loading of class I MHC molecules by interference with the transporter associated with antigen processing (TAP), resulting in the down-regulation of class 1 MHC expression on the surface of HSV infected cells.[28,37] To confirm that removal of ICP47 from JS1/ICP34.5 increased class I MHC expression on infected cells, an experiment in MDA-MB-231 human breast adenocarcinoma cells was performed. Cells were infected with either JS1, JS1/ICP34.5− or JS1/ICP34.5−/ICP47− at an MOI of 1 and 16 h later FACS analysis performed staining for class I MHC. This showed that, as expected, removal of ICP47 increased levels of class I MHC on the surface of infected cells as compared to cells infected with JS1 or JS1/ICP34.5− (Figure 5).
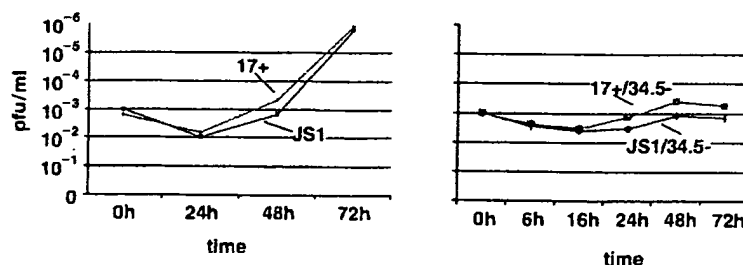

Figure 3 JS1/ICP34.5− has growth properties typical for an HSV ICP34.5 deleted virus. 3T6 cells were infected with either wild-type virus strains 17+ or JS1 (left) or ICP34.5 deleted strains 17+/ICP34.5− or JS1/ICP34.5− (right) and virus growth assessed over time.
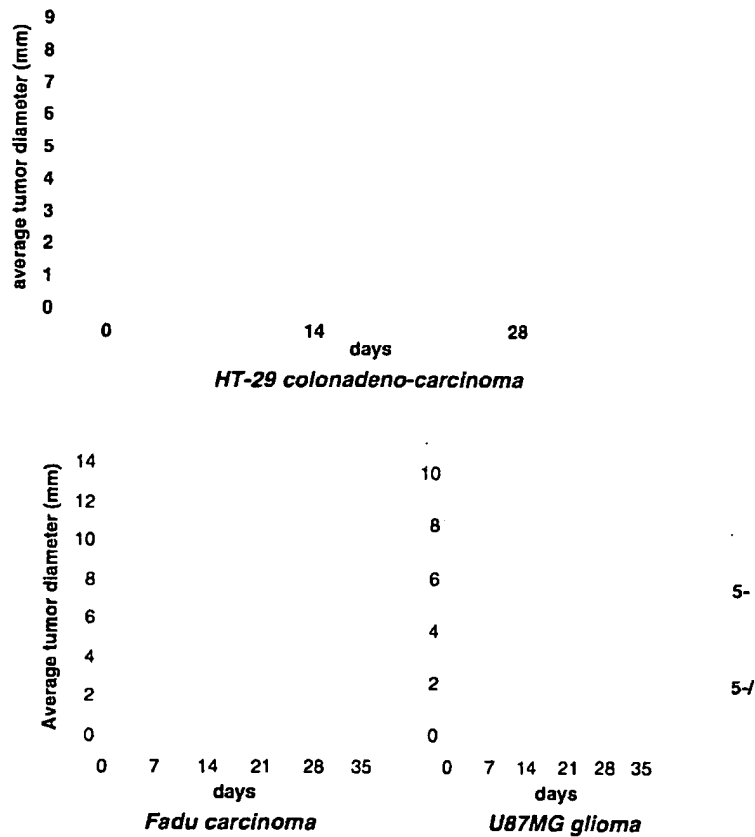
Figure 4 *Deletion of ICP47 improves anti-tumour effects in vivo. The indicated xenograft tumours were generated in the flanks of nude Balb/c mice. When tumours had reached approximately 0.5 cm in diameter, tumours were injected every other day a total of three times (arrows) with 50 μl of a 1 × 10⁸ pfu/ml stock of the indicated virus and effects on tumour growth measured. N=10 animals/group. Statistical analysis was carried out comparing the two virus-treated groups at the last time point using the unpaired Student's t-test. This gave the P values indicated, demonstrating statistical significance in each case.*
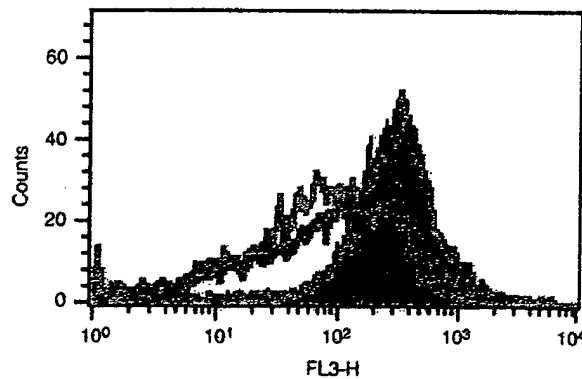

Figure 5 *Deletion of ICP47 prevents down-regulation of class I MHC molecules on the surface of infected cells. FACs analysis was performed on MDA-MB-231 human breast adenocarcinoma cells staining for class I MHC molecules (see Methods). Purple – mock infected cells; Blue – JS1/ICP34.5 –/ICP47 – infected cells; Red – JS1/ICP34.5 – infected cells; Green – JS1 infected cells.*

Incorporation of the gene encoding GM-CSF improves tumour reduction, particularly in non-injected tumours

Oncolytic HSV has been generally proposed for direct intra-tumoral injection where the greatest anti-tumour effect would be anticipated to be in the injected tumour.

We aimed to improve on the previously reported anti-tumour immune effects of ICP34.5 deleted HSV by incorporation of a cytokine gene. This was aimed at improving the systemic anti-tumour effects and also the degree of protection against later tumour growth. GM-

Injected tumours

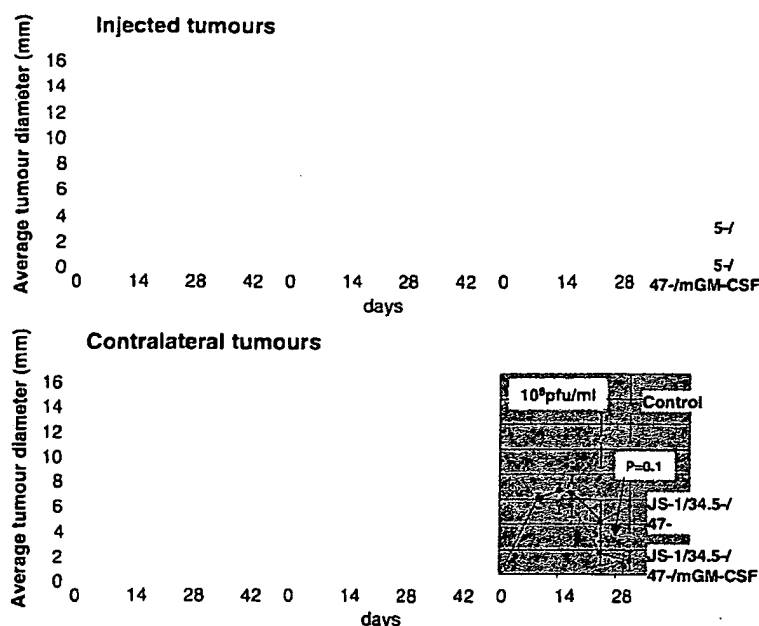

Contralateral tumours

Figure 6 Expression of GM-CSF improves tumour reduction, particularly in non-injected tumours. A20 lymphoma tumours were generated in both flanks of Balb/c mice. When tumours had reached approximately 0.5 cm in diameter, tumours on one flank were injected every other day a total of three times (arrows) with 50 μl of either a 1 x 10⁶ (left panels), 1 x 10⁷ (middle panels) or 1 x 10⁸ (right panels) pfu/ml stock of the indicated virus, or with vehicle (control). Effects on tumour growth were then measured. N=10 animals/group. Statistical analysis was carried out to compare the high-dose-virus-treated groups at the last time point using the unpaired Student's t-test. This gave the P values indicated demonstrating a statistically significant effect of the inclusion of GM-CSF in the contralateral but not the injected tumour groups.

CSF was chosen as it is well tolerated in man and promising anti-tumour effects have been demonstrated when delivered by other means.

The genes encoding human or mouse GM-CSF, driven by the CMV promoter were therefore inserted into the JS1/ICP34.5/ICP47— backbone in place of both the two genes encoding ICP34.5. GM-CSF expression was confirmed by ELISA both *in vitro* (>120 ng/5 x 10⁵ BHK cells 24 h after infection at MOI=0.1) and in mouse tumour extracts (>500 ng GM-CSF/mg total protein 24 h after injection with 5 x 10⁶ pfu of virus into 0.5–1 cm diameter A20 lymphoma tumours in mouse flanks). The virus carrying the mouse version of the GM-CSF gene was tested in syngeneic tumour models in Balb/c mice in comparison to the virus without GM-CSF. Tumours were induced in both flanks of mice, one of the flanks injected three times with the respective vector at three different doses, and effects on the tumours in both flanks observed (Figure 6). This demonstrated a dose-related anti-tumour effect in the injected tumour which was similar between the viruses with and without GM-CSF. However in the contralateral, non-injected tumour, while a dose-related anti-tumour effect was observed with both viruses, the degree of tumour shrinkage (Figure 6), and the number of tumours cured, was greatly improved by the addition of GM-CSF.

GM-CSF enhances the anti-tumour immune response

To confirm that the expression of GM-CSF enhanced the A20-specific immune response, interferon γ (IFN-γ) levels were assessed following *in vitro* restimulation of splenocyte cultures from tumour bearing mice, either mock injected or injected with the virus +/−GM-CSF.
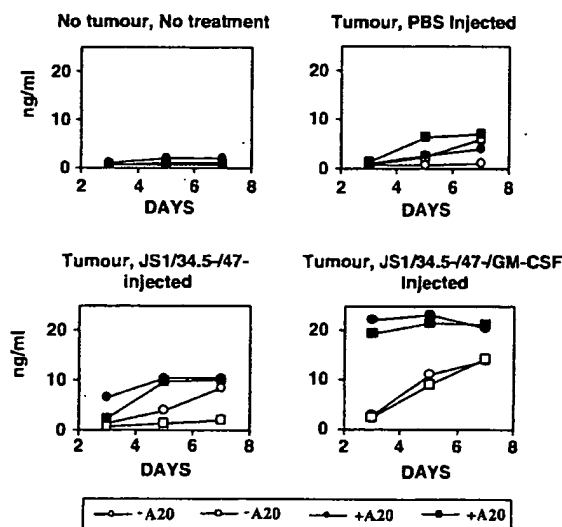

Figure 7 GM-CSF enhances the anti-tumour immune response. Pairs of mice were either left untreated (top left) or A20 lymphoma tumours generated. Tumours were then mock injected three times with PBS or tumours cured by injection three times with 50 μl of a 1 x 10⁸ pfu/ml stock of JS1/34.5−/47− or JS1/34.5−/47−/GM-CSF, respectively. Two weeks after the last injection, splenocyte cultures were prepared either in the presence (black symbols) or absence (open symbols) of exogenously added A20 cells. The concentration of IFN-γ in samples of culture supernatants collected at the times indicated were then measured by ELISA.

The concentration of IFN-γ in samples of culture supernatants collected over time were then measured by ELISA (Figure. 7). This demonstrated that considerably

increased levels of IFN-γ were generated from spleno-cytes in response to A20 cells when animals had been treated with JS1/ICP34.5−/ICP47−/mGM-CSF as com-pared to the equivalent virus without mGM-CSF (JS1/ICP34.5−/ICP47−).

Animals in which tumours have previously been cleared are protected against further tumour cell challenge

Having demonstrated that a potent, GM-CSF-enhanced, anti-tumour immune response is generated with JS1/ICP34.5−/ICP47−/mGM-CSF, it would be anticipated that protective immunity may have been induced against further tumour cell challenge. To test this, mice in which tumours had previously been cleared were challenged with 1 × 10⁶ tumour cells, either in the flank or via the tail vein, and any tumour growth observed. Control animals in which tumours had not previously been cleared developed tumours either in the flank or liver (flank or tail vein tumour cell administration respectively; see Figure 8), whereas no tumours were generated in any animal in which the tumours had been cleared. Animals remained tumour free for at least 6 weeks after tumour cell administration and were protected against tumour cell challenge for at least six months. Thus, tumour treatment with JS1/ICP34.5−/ICP47−/mGM-CSF in-duces protective anti-tumour immunity in mice which protects against further tumour cell challenge.

Pre-existing anti-HSV immunity does not affect the anti-tumour effect

A significant proportion of the human population are sero-positive for HSV1 which might reduce the effec-tiveness of oncolytic HSV if used for tumour treatment in man. To assess any effects of pre-existing immunity to HSV on tumour treatment, tumours were generated in animals which had previously been immunized with wild-type HSV1. These were then treated with JS1/34.5−/47−/mGM-CSF. This demonstrated that tumours were effectively treated even following prior immuniza-tion with HSV, there being no detectable difference in the magnitude of the anti-tumour effect as compared to the previous experiments reported above (Figure 9). Thus, as previously reported for a previous version of oncolytic HSV,[38] prior immunity to HSV does not have a significant effect on the ability of the virus to treat tumours.

Discussion

A number of oncolytic viruses have been tested in the clinic, where they have generally proved to be well tolerated in the Phase I and II clinical trials conducted so far. Some indications of clinical efficacy have also been shown. However, it is evident that improvements to efficacy would be beneficial to increase effectiveness and also to reduce the doses of virus required. Reduction in
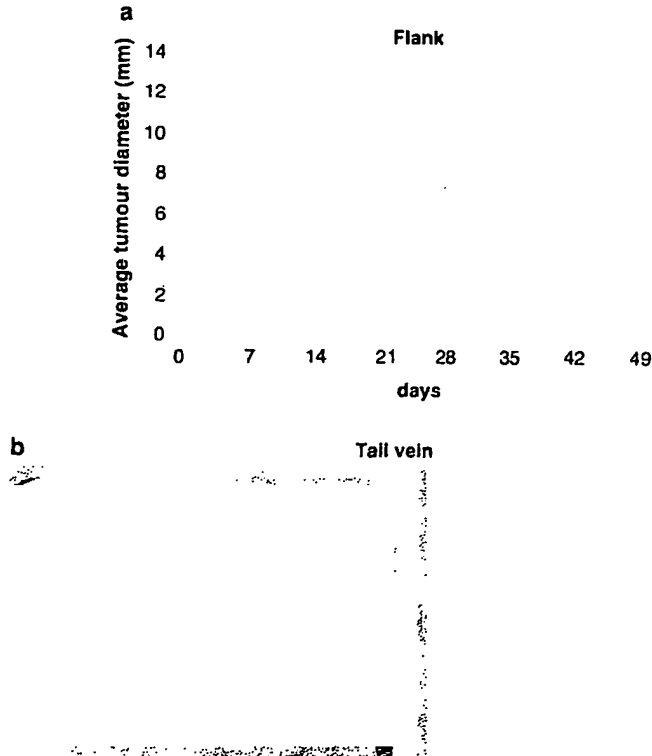

Figure 8 Animals in which tumours have been cleared are protected from tumour cell rechallenge. Balb/c mice in which A20 tumours had previously been cured (generated and treated as in Figure 6) were rechallenged either with 1 × 10⁶ A20 cells in the flank or by tail vein administration in comparison to control naïve animals. Growth of tumours in the flank over time (top) or in the liver 21 days post-tumour cell administration (bottom) were assessed.
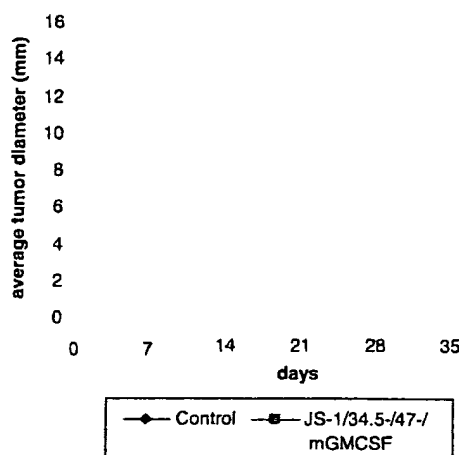
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Figure 9 Pre-existing anti-HSV immunity does not affect the anti-tumour effect. Balb/c mice were first immunized with wild-type HSV1 (see Methods). A20 lymphoma tumours were then generated in the flank. When tumours had reached approximately 0.5 cm in diameter, tumours were injected every other day a total of three times (arrows) with 50 μl of a 1 × 10⁸ pfu/ml stock of JS1/ICP34.5−/ICP47−/mGM-CSF, or with vehicle (control). Effects on tumour growth were then measured. N=10 animals/ group.

dose would potentially benefit not only the patient, but would also minimize production issues which have proved to be problematic with, eg oncolytic adenovirus during scale-up for later stage clinical testing.

In this paper, we have built on previous work to improve the potency of oncolytic HSV. We aimed to boost both the tumour-selective replicative capability of the virus and also its immune stimulating ability to provide a multi-modal cancer therapy incorporating both direct virus replication-mediated cell lysis and the induction of an immune response to the antigens released. Such a virus would, apart from having a direct oncolytic effect, provide an *in situ*, patient-specific, anti-tumour vaccine. This, when administered into a tumour, would be anticipated to have effects not only on the treated tumour but also a systemic immune effect which may be beneficial in the treatment of metastatic disease.

In order to develop oncolytic HSV with greater tumour-selective replicative ability, we tested two clinical isolates of HSV for their ability to replicate in and kill human tumour cell lines. It was anticipated that recent clinical isolates might be more effective than the previously tested serially passaged laboratory strains. This proved to be the case, and the recent clinical strain of HSV1, JS1, was chosen for further development. The genetic basis of the improved cell killing properties of JS1 as compared to the reference strain (strain 17+) has not been fully explored, but restriction profiling suggests the genome to be generally similar to strain 17+, the only fully sequenced strain of HSV1. Of the regions of JS1 so far sequenced, a number of small differences to the 17+ sequence were identified and it seems likely that the overall improved properties of the virus result from multiple small differences scattered throughout the genome, none of which individually are responsible for the effects seen. Complete sequencing of the JS1 genome is currently underway.

Having identified a more potent strain of HSV than had previously been used for development as an

oncolytic agent, we deleted the genes encoding ICP34.5 to provide tumour-selective replication, and then also included a previously reported mutation resulting in the expression of the US11 gene as an IE rather than on L gene to further increase replication. Expression of US11 blocks phosphorylation of PKR which results in enhanced replication of ICP34.5 deleted viruses in tumour cells without compromising safety.[27,33] When tested in mouse xenograft tumour models, this mutation considerably enhanced the anti-tumour effect, as expected from previous work.[25] Increased expression of US11 is accomplished by deletion of the coding sequence for ICP47. Since ICP47 usually inhibits antigen presentation in HSV infected cells,[28] this deletion would be expected to increase levels of antigen presentation in infected tumour cells. This should improve any anti-tumour immune response following intra-tumoral injection of the virus by two potential mechanisms. Firstly, tumour antigens would be expected to be expressed more effectively on the surface of infected tumour cells prior to cell death. Secondly, ICP47 expressed following infection of professional antigen presenting cells, such as dendritic cells in the vicinity of the tumour would not then be expected to inhibit their antigen presenting capabilities. As expected, we observed increased class I MHC expression on the surface of human cells infected with JS1/ICP34.5−/ICP47−, compared to the equivalent virus in which ICP34.5 alone was deleted. However, direct effects of ICP47 deletion on the immune stimulating capabilities of the viruses in tumour models were not tested as ICP47 has previously been shown not to function in mice.

Finally, to improve the potential for an anti-tumour immune response further, we inserted the gene for GM-CSF. While a number of cytokines have been considered for use in anti-tumour therapy (including the use of GM-CSF or IL12 in oncolytic HSV vectors[39–43]), GM-CSF has generally, and most reliably, given the best results when tested in comparison to other cytokines in pre-clinical models. Clinically, for cancer treatment, indications of efficacy have also been seen when GM-CSF has been delivered by a number of means (eg using engineered, re-injected primary tumour cells — 'GVAX'; eg reference 44). Additionally, while other cytokines have given promising results in clinical and pre-clinical cancer studies (notably IFN-α, IL12 or TNF α), GM-CSF is already used in routine clinical practice and is generally well tolerated at high doses without significant side effects. When the gene for GM-CSF was inserted into the ICP34.5/ICP47 deleted version of JS1, an enhanced anti-tumour effect was seen in artificially induced tumours in immune competent mice where significant effects on both injected and uninjected tumours were seen. Mice were also then protected from re-challenge with tumour cells. An enhanced tumour-specific immune response with the virus encoding GM-CSF was also demonstrated.

Overall therefore, we describe a highly potent oncolytic version of HSV which is designed to also stimulate an enhanced anti-tumour immune response by the inclusion of the gene encoding GM-CSF. The virus would therefore be anticipated to have a potent oncolytic anti-tumour effect if used for cancer treatment in man, and also provide an *in situ*, patient-specific tumour vaccine, following intra-tumoral injection and the liberation of tumour antigens. The virus should therefore have effects

on both injected and uninjected tumours enhanced by a significant immune effect, as proved to be the case in mice. This, combined with the previous clinical data accumulated for oncolytic viruses generally, suggests that JS1/ICP34.5–/ICP47–/GM-CSF is a highly promising candidate for clinical development as an anti-cancer agent.

JS1/ICP34.5–/ICP47–/GM-CSF has received approval from the Gene Therapy Advisory Committee (GTAC) and the MCA in the UK to enter Phase I clinical testing by direct intra-tumoral injection in a number of tumour types. This trial is currently underway.

Methods

Viruses

Viruses were grown in BHK 21 C13 cells (ECACC 85011433) in DMEM containing 10% foetal calf serum (Gibco). HSV1 strains, JS1 and BL1, were isolated by taking a swab from a cold sore of otherwise healthy volunteers. JS1 and 17+ viral DNA were obtained using DNAzol™ (Helena Biosciences, Sunderland). For deletion of the ICP34.5 gene from JS-1 and 17+, a plasmid pΔ34.5 CMV GFP was constructed consisting of a Sau3A fragment of HSV-1 17+ (nucleotides 123462–126790) inserted into the BglII site of plasmid pSP72 (Promega, Madison, USA). A NotI fragment (nucleotides 124948-125713), encoding ICP34.5, was then removed. A BbsI/NruI fragment from pCDNA3CMVGFP, GFP under the control of the CMV promoter and bGH polyA signal, was inserted into the NotI site in pΔICP34.5. pCDNA3CMVGFP was produced by the insertion of a HindIII/NotI fragment, containing EGFP from the plasmid pEGFP-N1 (Clontech, Palo Alto, USA), into the NotI site of pCDNA3 (Invitrogen, Groningen, Netherlands). pΔICP34.5 CMV GFP was used to delete ICP34.5 in both JS1 and 17+ by standard techniques giving viruses 17+/34.5– and JS1/34.5–. To insert human and mouse GM-CSF genes, the GFP gene in pΔICP34.5 CMV GFP was replaced with either the human or mouse GM-CSF gene, and these plasmids used to remove the GFP from JS1/34.5– giving viruses JS1/34.5–/hGM-CSF and JS1/34.5–/mGM-CSF. A virus was also constructed in which the CMV-GFP-pA cassette was removed from JS1/34.5– by recombination with 'empty' ICP34.5 flanking regions giving JS1/34.5–/w.

To delete ICP47, a PCR fragment encoding nucleotides 145570–146980 of the 17+ genome (primers GCATCGATCTTGTTCTCCGACGCCATC and GCAAGCTTGCTCCCCCCCGACGAGCAGGAAG) was inserted into the HindIII/SalI site of pBluescript (pBSK SK, Stratagene, USA) giving p47US. A second PCR fragment encoding nucleotides 143675–145290, (primers TCTAGAGGGTTCGATTGGCAA TGTTGTCTCCCG and TTAACGATCGAGTCCCGGGTACGACCATCACCCG) was subcloned into pGemT Easy (Promega, USA). An EcoR1 fragment from this plasmid was then inserted into the SpeI site in p47US generating pΔ47. Both PCR fragments were sequenced. The BbsI/NruI fragment from pCDNA3 CMV GFP was then inserted into the HindIII site in pΔICP47.

pΔICP47 CMV GFP was then recombined with JS1/34.5–/w, JS1/34.5–/hGM-CSF and JS1/34.5–/mGM-CSF giving viruses with a GFP insertion so as to replace

the ICP47 gene. GFP was then removed from each of these using pΔICP47 giving JS1/34.5–/47–, JS1/34.5–/47–/hGM-CSF and JS1/34.5–/47–/mGM-CSF.

GM-CSF genes

The human GM-CSF gene was cloned from an IMAGE clone 2340997/5808-K14 (UK HGMP Resource Centre) using primers CTGAAGCTTATGTGGCTGCAGAGCCTG and TGGCTCGAGTCACTCCTGGACTGGCTC. The mouse GM-CSF gene was cloned by RT PCR from RNA isolated from mouse lung tissue using primers TCCTGAGGAGGATGTGGCTG and CTGGCCTGGGCTT CCTCATT. Both the human and mouse GM-CSF genes were then sequenced.

ELISA for GM-CSF expression

A20 tumours were induced in Balb/c mice and tumours injected with 5×10^6 pfu of vector. One day after vector administration, tumours were homogenized and levels of GM-CSF determined using a commercial ELISA kit (Amersham Biotrak). ELISAs were also performed on culture media following growth of GM-CSF expressing vectors in BHK cells.

Restriction profiling

Approximately 2.5×10^5 cells were infected at an MOI of 10 with the appropriate virus (HSV1 stain 17+ or JS1 or HSV2 strain HG52), $5\,\mu Ci$ inorganic ^{32}P added to the culture medium, and incubated at 37°C for 48 h. DNA was then harvested by the addition of 500 μl of 5% SDS/well, incubated at 37°C for 5 min followed by phenol extraction and ethanol precipitation. DNA was restriction enzyme digested overnight and then electrophoresed on a 1% agarose gel which was dried down prior to exposure to film.

In vivo tumour models

Tumours were induced in the flanks of Balb/c or Balb/c nude mice by subcutaneous injection of 2×10^6 of the appropriate tumour cells and tumours allowed to develop to an average diameter of approximately 0.5 cm. prior to vector administration. In all cases 10 animals/treatment group were used. Graphs show the average tumour diameter and the standard error for each group. Any animal showing adverse effects was terminated in accordance with local rules, as was the case for all the animal experiments described. Statistical analysis was performed using the unpaired Student's t-test.

Assessment of immune responses

Spleens were harvested 2 weeks after the final virus injection into A20 lymphoma tumours (see above) by which time tumours had been cured in most mice. 2×10^7 splenocytes were cultured in 5 ml RPMI containing 10% heat-inactivated serum. Cultures were supplemented, where necessary, with 5×10^5 A20 cells which had been previously treated for 1 h with 25 μg/ml mitomycinC (Sigma), and then extensively washed. Samples of supernatant were removed and stored at –20°C. The concentration of mouse IFN-γ in the super-

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natants was assessed using a Ready-SET-Go kit (Insight Biotechnology Ltd, Wembley, UK).

FACS analysis to assess MHC expression

Human breast adenocarcinoma cells (MDA-MB-231, ECACC [Salisbury, UK]) were infected at 90% confluency with the viruses JS1, JS1/ICP34.5$^-$ or JS1/ICP34.5$^-$ / ICP47$^-$ at a MOI of 1 or mock infected. 1×10^5 cells were washed with 15 mM EDTA, 30 mM NaN_3, 1% BSA in PBS and stained on ice for 30 min with biotin conjugated mouse monoclonal anti-HLA-ABC W6/32 (Serotec, Kidlington, UK). Cells were then washed three times as above and stained on ice for 30 min Streptavidin-RD670™ (Life Technologies). Ten thousand events were collected and analysed on a FACSCalibur with CellQuest software (Becton-Dickinson, Oxford, UK).

Prior immunization of mice

To induce an anti-HSV immune response, a similar procedure to previous work was used.[38] Balb/c mice were immunized i.p. with 5×10^3 pfu wild-type HSV1 (17+) in PBS 5 weeks before tumour induction. Three weeks later (2 weeks before tumour induction) this dosing was repeated.

Intra-cerebral dosing of mice

Three-week-old female Balb/c mice were anaesthetized and inoculated directly through the skull into the right hemisphere using an insulin-type syringe with 20 μl of a 1×10^3, 1×10^4, or a 1×10^5 pfu/ml stock of JS1/ICP34.5−. Following recovery, any signs of adverse effects were monitored.

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